AD_____

Award Number: DAMD17-03-1-0193

TITLE: Biological Effects of Activating Distinct ErbB Receptor
        Dimers in Polarized Growth Arrested Epithelial

PRINCIPAL INVESTIGATOR: Marissa Moore

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
                           Cold Spring Harbor, New York 11724

REPORT DATE: September 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20050407 116

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (16 Aug 2003 – 15 Aug 2004)

4. TITLE AND SUBTITLE
Biological Effects of Activating Distinct ErbB Receptor Dimers in Polarized Growth Arrested Epithelial

5. FUNDING NUMBERS
DAMD17-03-1-0193

6. AUTHOR(S)
Marissa Moore

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York   11724

E-Mail: moore@cshl.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland   21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

ErbB family of receptor tyrosine kinases have been implicated in human breast cancers. In particular ErbB2 is over expressed in 25-35% of all breast cancers . We can selectively activate a particular receptor using a chimeric system and synthetic ligand. We combine this system with a three-dimensional cell culture system, which allow cells to grow into mammary acini like structures in vitro. We have shown that activation of ErbB2 induces a change in cell polarity and re-initiates proliferation in 3D structures. Using these systems I propose to investigate how activation of ErbB2 disrupts epithelial cell polarity and disrupts proliferation control. In particular, I am interested in investigating how the Rho family of small GTPases (RhoA, Rac1, CDC42) and Par complex regulates ErbB2-induced transformation of human mammary epithelial cells. We have generated the reagents necessary to investigate this question. Preliminary experiments have shown that ErbB2 activation cooperates with RhoN19 expression in 3D to increase disruption of acini architecture. We also observed that over expression of Par6 FL in MCF-10A cells promotes EGF independent proliferation. We will build on these observations and others to help us determine the role played by these proteins in ErbB2 induced transformation.

14. SUBJECT TERMS
ErbB, polarity, proliferation, mammary epithelial cell biology, three dimensional

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction: Malignant breast cancer appears to develop over time from mammary epithelial cells that have been transformed. These transformed cells form early lesions that are characterized by uncontrolled proliferation and loss of epithelial architecture. Currently, early lesions are defined using histological techniques and the molecular features that distinguish different histological types are not known. We do know that certain oncogenes and tumor suppressors are implicated in breast cancer but the specific molecular mechanisms that initiate transformation and contribute to histopathology of the tumor remains to be elucidated.

ErbB receptor network and breast cancer: ErbB receptors are a family of receptor tyrosine kinases (RTKs) that are important during normal development and tumorigenesis of the mammary gland. Many biological processes are regulated by ErbB receptors such as cell division, migration, adhesion, differentiation, morphogenesis and prevention of apoptosis[1]. There are four members in this family, ErbB1 (EGFR, HER1), ErbB2 (HER2/Neu), ErbB3 and ErbB4. Over-expression of ErbB is associated with breast, ovary, brain and prostate cancers. ErbB2 is over-expressed in 25-30% of breast cancers. ErbB2 gene amplification and overexpression also correlates with a poor clinical prognosis[2, 3]. There has been recent evidence that ErbB2 over-expressing tumors also co-overexpress ErbB3[4].

Rho GTPase family and cancer: Rho proteins can cycle between GTP and GDP bound states, which are effected by a variety of different regulator molecules that either promote or inhibit GTP binding. They are categorized into three different subgroups based on similarity to RhoA, Rac1 and Cdc42, and proteins that lack GTPase activity. Rho GTPases are over-expressed in human breast, colon, lung and pancreatic cancers [5]. Specifically RhoA, Rac1 and Cdc42 are over-expressed in breast cancer [6]. Since then, there have been no reports mutated Rho proteins in tumors. The evidence suggests that it is cycling of GDP/GTP that is important for transformation [7, 8]. Other studies show that Rac activity is increased in breast cancer cell lines[5, 9]. The accumulating evidence suggests that it is the deregulation of the proteins that correlates with tumor progression and poor prognosis[5].

Epithelial cell polarity: Mammary epithelial cells line the ducts of the mammary gland and possess an apical-basal polarity as defined by their ability to localize their tight junctions, gap junctions and adherens junctions in discrete localization along the apical-basal axis. Establishment of apical-basal cell polarity requires the concerted effort of protein complexes and other localized proteins. Recent studies have identified three protein complexes namely, the Par complex, Scribble complex, and the Crumbs complex that act in a cooperative manner to establish apical-basal polarity[10-12]. Recent studies demonstrate that in addition to being part of the Par complex, Par6 also interacts with the crumbs complex and the Scribble complex suggesting that Par6 may play and important role in coordinating the interaction with other polarity complexes [13].

Cell culture system: Our lab combines a unique system to study the inducible effects of ErbB receptors with a three-dimensional cell culture system. ErbB receptors have the ability to homo and heterodimerize generating a complex signaling system. Our lab has circumvented this problem by using a chimeric ErbB receptor. The chimera can bind to synthetic ligand, dimerize, phosphorylate and generate downstream signals. This system allows us to specifically activate the receptor of interest. [14]. We are also culturing mammary epithelial cells (MECs) on a matrix abundant in collagen and laminin (Matrigel). The cells can grow from a single cell to into 3D acini-like structures with a single layer of epithelial cells surrounding a luminal space. These 3D acini structures have characteristics similar to the resting epithelium of mammary glands, such as low proliferation rate and organized architecture. Cell lines have been established that express chimeric ErbB1 (B1) and ErbB2 (B2) chimeras using a retroviral expression system. We have shown that activation of ErbB2 cells induces multilayering, changes in cell polarity and re-initiates proliferation [15].

4

Using the 3D cell culture approach and the ErbB receptor activating systems I propose to investigate how activation of ErbB2 disrupts epithelial cell polarity and disrupts proliferation control. In particular, I am interested in investigating how the Rho family of small GTPases (RhoA, Rac1, CDC42) regulates ErbB2-induced transformation of human mammary epithelial cells.

In addition, to their role in signaling pathways regulating proliferation and migration, Rho family members are known to play important roles in regulating epithelial cell polarity for instance, Rac and CDC42 are part of the Par protein complex and play critical roles during establishment of epithelial cell polarity. I propose to investigate how ErbB2, Par complex and the Rho family of proteins interact with each other to effect transformation of human mammary epithelial cells.

Body:

Generation of MDCK cells expressing Cdc42N17 and ErbB1 or ErbB2. As outlined in **task three, I** utilized epithelial cells lines that were a gift from James Nelson's lab that express tetracycline regulated dominant negative Cdc42N17. Using these cells I generated two cell lines that stably express either ErbB1 or ErbB2 chimeric proteins. In order to determine spindle orientation it is necessary to seed the cells on a porous filter at a high confluency. The cells will begin to establish apical-basal cell polarity and growth arrest. However, once the cells were growth arrested we were not able to induce expression of Cdc42N17 by removal of tetracycline from the media. Although MDCK cells are dog intestinal epithelial cells, these cells were initially chosen because they are ideal for investigating aspects of epithelial cell polarity. However, given that I faced technical challenges, I chose not to optimize these cells and instead chose to use human mammary epithelial cells for my proposed studies.

Generation of Adenoviral vector expressing Cdc42N17, RhoN19 and RhoAWT: We obtained the expression plasmids, pCG CDC42N17 pCDNA RhoAWT and pGBT8 RhoN19 from Linda Van Alest's lab. Since, I am interested in inducing expression of genes in proliferation-arrested cells. We decided to adapt an inducible adenoviral expression system to carry out my studies. We utilized a vector backbone (obtained from J. Chernoff) and generated adenoviral expression vectors that allow for tetracycline regulated protein and IRES GFP expression (Figure 1). I successfully generated a Cdc42N17, RhoN19 and RhoWT expression vectors. Adenovirus was successfully generated with Cdc42N17 and RhoN19. We a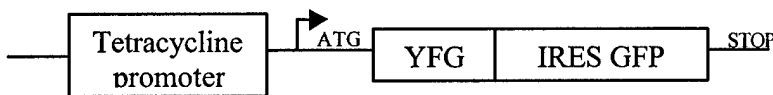lso obtained existing adenovirus RacN17, RhoN19 and control GFP from J.Chernoff. We utilized these viruses for immediate experimentation.

Figure 1. Adenoviral expression vector

Determination of optimal adenoviral infection conditions and cell line: In order to answer the question, "do dominant negative Rho GTPase affect ErbB2 induced phenotype", we needed to establish the adenoviral system. We had a choice of different cell lines. Madin-Darby Canine Kidney epithelial cells (MDCK), human mammary epithelial cells 184 A1 and human mammary epithelial cells MCF-10A. 184A1 cells proved not to be well suited for generation of polarized epithelial monolayers.. They were also not optimally suited for 3D cultures. Although MDCK cells are well suited for generation of polarized monolayers, I was unable to obtain high infection efficiency in polarized growth arrested monolayers. However, MCF-10A cells were easily infected with adenovirus and had robust protein expression with low viral titers (30 particles/cell) (Figure1 and 2). I decided to use MCF-10A cells for subsequent experiments for 4 reasons: (1) they have the ability to generate acini structures in 3D, (2) they can be infected easily, (3) they are derived from human breast tissue making them relevant to breast cancer and (4) our lab is well experienced in using MCF-10A cells as a tool.

Expression of Rho family proteins during formation of mammary acini: For these experiments MCF-10A cells were plated on 3D growth conditions. The cells were infected on Day 2 (d2) and the acini were allowed to develop for 4 – 10 days. The changes in morphology were noted. Both RhoN19 and RacN17 had an effect on the developing acini structures (Figure 4A). The cells lost the ability to form organized acini structures, allowing for protrusions and blebbing formation. This data suggests that the cell lost their ability to regulate establishment of cell polarity.

Expression of Rho family proteins in fully formed mammary acini: For these experiments MCF-10A cells were plated on 3D growth conditions. The cells developed into acini structures and were infected on Day 8 (d8) and allowed to express protein for 4 days. Neither RacN17 nor RhoN19 appeared to affect the maintenance of the acini structures. The infected acini were indistinguishable from the control (Figure 4B). Together these data suggests that Rho and Rac are important for development but not in maintenance of the acini structure.

Activation of ErbB2 in conjunction with RhoN19, RacN17 and Cdc42N17 expression in D12 structures: The Rho family of GTPases have been shown to be activated via Ras, which is a primary effector pathway of ErbB2. Activation of ErbB2 re-initiates proliferation and induces disruption of 3D acini-like structures. We investigated whether interfering with the Rho family inhibits the ErbB2 induced phenotype. We have preliminary data that was gathered using the dominant negative Rho GTPases, RacN17, RhoN19 and Cdc42N17 the data is summarized in Table 1. Expression of RacN17 had no effect of ErbB2 induced phenotype. Expression of Cdc42N17 induced disruption of the structures without activation of ErbB2. Interestingly there seems to be a corporative effect between RhoN19 expression and ErbB2 activation. The structures have change in morphology, and their architecture is disrupted with protrusions and blebbing (Figure 5). As determined by Ki-67, ErbB2 is still able to re-initiate proliferation in the presence of RhoN19 expression. These observations will be followed by more extensive experimentation to characterize these phenotypes.

Generation of Estrogen Receptor inducible Par3 mutations and Myc retroviral expressing MDCK cell lines: As outlined in **task two,** I began to generate cell lines that retrovirally expressed flag-tagged Estrogen receptor (FER) Myc, (the backbone plasmid was a gift from Bill Tansey). Expression of this protein was problematic, and I was unable to monitor the effect of activation using tamoxifen. Concurrently, I generated FER vectors expressing Par3 to disrupt tight junction formation. Truncations were made after PDZ domain 1 aa1-358, PDZ domain 2 aa1-546 and PDZ domain 3 aa1-676. Virus was generated and stable cell lines were made. We were able to detect protein expression but unable to determine if the truncations were active upon addition of tamoxifen. Therefore, I changed my strategy to utilize both the adenoviral system and a non-inducible retroviral expression system in MCF-10A cells.

Generation of Par3 and Par6 mutations in adenoviral and retroviral vectors: The ultimate goal is to generate both adenoviral and retroviral vectors containing a panel of Par3 and Par6 mutations. To date the Par3 truncations, Par6 full length and Par6 N-Term aa1-150 are in adenoviral vectors. Par3 full length, Par6 Full length and Par6 C-Term aa140-345 are in retroviral vectors. MCF-10A cell lines were generated that stably express Par3 FL, Par6 FL and Par6 CT. Cloning difficulties prevented the entire panel in both adenoviral and retroviral vectors to be made simultaneously and are in progress.

Full-length Par6 expression induced EGF independent growth on 2D: Under normal cell culture conditions MCF-10A cells require EGF to grow. The cells will significantly slow their growth in EGF depleted media. The FL par6 expressing cell line proliferated rapidly in EGF free media when compared to control vector, Par6 CT and parental MCF-10A cells Figure 6. This is a major observation that we will continue to follow and determine if EGF independent growth can occur in the 3D cell culture system. Par6 is a major component of the par complex and new information suggests that plays

6

an even more essential role interacting with other components of polarity complexes such as crumbs and Lgl proteins. It will be interesting to see if MCF-10A par6 cells can grow in 3D cultures without EGF.

Full length Par3 and Par3 PDZ3 truncation induces acid production: Another interesting observation was noted with Par3 Full length MCF-10A cells. While proliferation and polarity seem to be the same as parental cell lines the media rapidly changes color indicating an increase metabolism. This observation also occurs when the PDZ3 truncation is introduced to growth arrested acini. Again proliferation was not effected as determined by Ki-67 staining. In both cases we consistently see a color change in the media and this needs to be investigated further.

Future Directions: Once I have generated conclusive observation utilizing these reagents I can then begin to address the questions in **task 1** and introduce ErbB1-ErbB2 heterodimers. These cell lines have already been generated by the lab and can be used to further my investigations.

Adenoviral Vector			
Protein Expressed	**Successful Virus Generation**	**Phenotypic observation in D12 3D structures**	**Activation of ErbB2 phenotype in D12 3D structures**
Cdc42N17	Yes	Disruption of architecture	Disruption of architecture
*RacN17	Yes	None observed	None observed
*RhoN19	Yes	None observed	Disruption of architecture
RhoWT	No	Not known	Not known
Par3 FL	No	Not known	Not known
Par3 PDZ1	No	Not known	Not known
Par3 PDZ2	Yes	None observed	None observed
Par3 PDZ3	Yes	Changes media color	Changes media color
Par6 FL	Yes	Not known	Not known
Par6 CT	No	Not known	Not known
Par6 NT	Yes	Not known	Not known
Retroviral Vector			
Protein Expressed	**Successful Virus Generation**	**Phenotypic observation in 2D**	**Activation of ErbB2 phenotype in 2D**
Par3 FL	Yes	Changes media color	Not known
Par6 FL	Yes	EGF independent proliferation	Not known
Par6 CT	Yes	None observed	Not known
Par6 NT	No	Not known	Not known

* Gift from Jonathan Chernoff FCCC.

Table 1. Summary of viral constructs generated, phenotypic observations in 3D cell culture system and traditional 2D cell culture system.

Key Research Accomplishments:

- Determined that adenoviral mediated protein expression is a useful system to inducibly express proteins of interest in growth arrested 3D acini structures.
- Standardized conditions of adenoviral infection in MCF-10A cell line in 2D and 3D
  - Observation RhoN19 and RacN17 disrupt the development of 3D acini but do not disturb maintenance of structure once they have been formed.
- Generation of adenoviral vectors, adenovirus and retrovirus.
  - Adenoviral Vectors:
    - Par6 N-terminal
    - Par6 C-terminal
    - Par3 truncation PDZ1
    - RhoA wild type
  - Adenovirus:
    - Par3 truncation PDZ2
    - Par3 truncation PDZ3
    - Cdc42N17
    - Par6 full length
  - Retrovirus:
    - Par3 truncation PDZ1 FER
    - Par3 truncation PDZ2 FER
    - Par3 truncation PDZ3 FER
    - Par6 full length MSCV IRES GFP
    - Par6 C-terminal MSCV IRES GFP
    - Par3 full length MSCV
- Determined conditions to express Rho family and activation of ErbB2 in 3D.
  - Observation that RhoN19 cooperates with ErbB2 to disrupt acini architecture.
- Generation of MCF-10A cell lines expressing Par proteins (see below)
- Identified the ability of Par6 to promote EGF independent cell proliferation in 2D

Reportable Outcomes:

Academic:

Successful defense of thesis proposal - March 2004

Oral presentation: Cold Spring Harbor Laboratory Student seminar - May 2004
                    Stony Brook University Student Seminar - April 2004

Poster presentation: Stony Brook University Genetic Program Symposium - January 2004

Development of reagents and cell lines:

Cell lines:

MCF-10A expressing Par3 full length
MCF-10A expressing Par6 full length
MCF-10A expressing Par6 C-Terminal
MDCK Cdc42N17 expressing ErbB1 chimera
MDCK Cdc42N17 expressing ErbB2 chimera

Conclusions:

First I determined that adenovirus was a viable system to introduce regulated protein expression in both traditional cell culture systems 2D, and in 3D mammary epithelial acini structures. Once the conditions were optimized I began to determine if the effect of ErbB2 can be affected by the introduction of dominant negative Rho GTPases. While ErbB2 activation alone re-initiates proliferation and disrupts cell polarity, we observed a cooperation between ErbB2 and RhoN19, which further disrupts 3D acinar architecture. It was also determined that RhoN19 and RacN17 interfered with the development of the acini however, they had no effect in developed structures. Many other reagents were generated to interfere with cell polarity. We did this in two systems. A retroviral mediated constitutively expressed MCF-10A cells and adenoviral inducible expression system. We observed that Par6 overexpression allows for EGF independent proliferation on 2D cell culture, we will next determine if the cells have the same ability in 3D culture. The generation of these reagents and cell lines will help us determine how ErbB2 transforms mammary epithelial cell acini structures.

References:

1.   Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.

2.   Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.* Science, 1989. **244**(4905): p. 707-12.

3.   Slamon, D.J., *Proto-oncogenes and human cancers.* N Engl J Med, 1987. **317**(15): p. 955-7.

4.   Siegel, P.M., et al., *Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer.* Embo J, 1999. **18**(8): p. 2149-64.

5.   Sahai, E. and C.J. Marshall, *RHO-GTPases and cancer.* Nat Rev Cancer, 2002. **2**(2): p. 133-42.

6.   Fritz, G., I. Just, and B. Kaina, *Rho GTPases are over-expressed in human tumors.* Int J Cancer, 1999. **81**(5): p. 682-7.

7.   Lin, R., et al., *A novel Cdc42Hs mutant induces cellular transformation.* Curr Biol, 1997. **7**(10): p. 794-7.

8.   Zondag, G.C., et al., *Oncogenic Ras downregulates Rac activity, which leads to increased Rho activity and epithelial-mesenchymal transition.* J Cell Biol, 2000. **149**(4): p. 775-82.

9.   Mira, J.P., et al., *Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 185-9.

10. Ohno, S., *Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity.* Curr Opin Cell Biol, 2001. **13**(5): p. 641-8.

11. Humbert, P., S. Russell, and H. Richardson, *Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer.* Bioessays, 2003. **25**(6): p. 542-53.

12. Lemmers, C., et al., *hINADl/PATJ, a homolog of discs lost, interacts with crumbs and localizes to tight junctions in human epithelial cells.* J Biol Chem, 2002. **277**(28): p. 25408-15.

13. Hurd, T.W., et al., *Direct interaction of two polarity complexes implicated in epithelial tight junction assembly.* Nat Cell Biol, 2003. **5**(2): p. 137-42.

14. Muthuswamy, S.K., M. Gilman, and J.S. Brugge, *Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers.* Mol Cell Biol, 1999. **19**(10): p. 6845-57.

15. Muthuswamy, S.K., et al., *ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini.* Nat Cell Biol, 2001. **3**(9): p. 785-92.
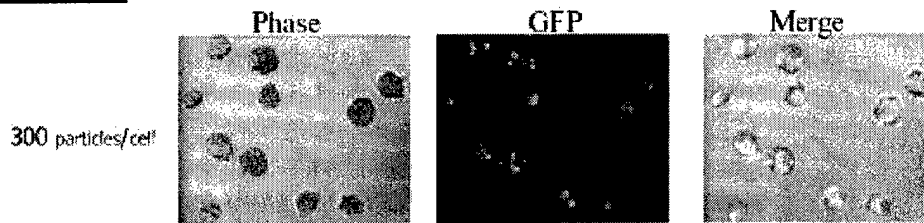
Appendix:

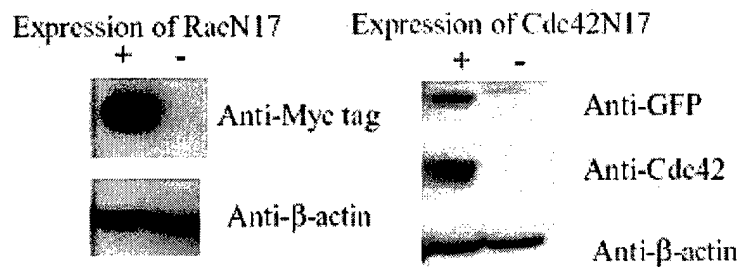Figure 2. Expression of control GFP Virus in D16 structures.

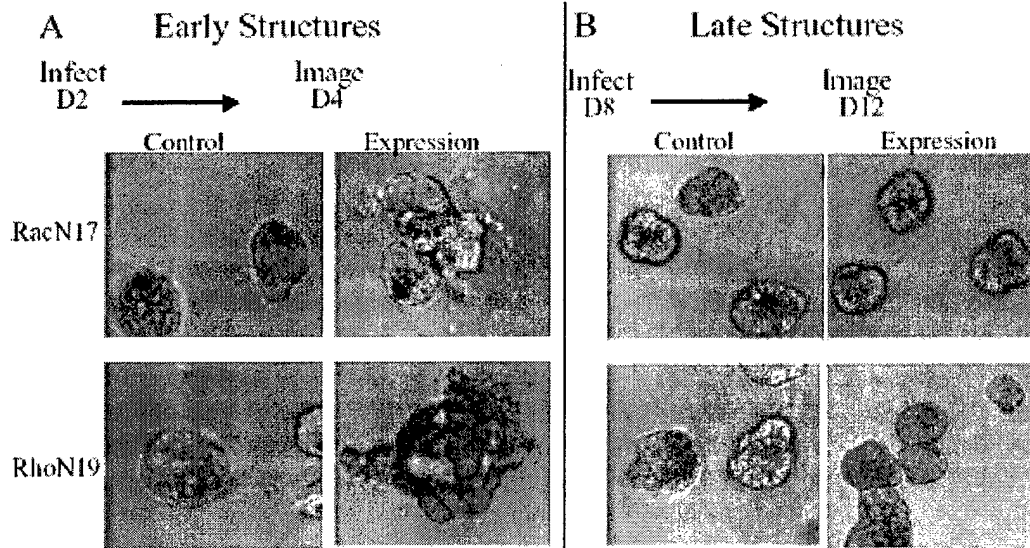Figure 3. Tetracycline regulated expression of cdc42N17 and RacN17 in 3D lysate.

Figure 4. Infection of RacN17 and RhoN19 during different days of acini development. Both proteins affect development of acini structures (A). However neither protein affects the structure once they are established (B).
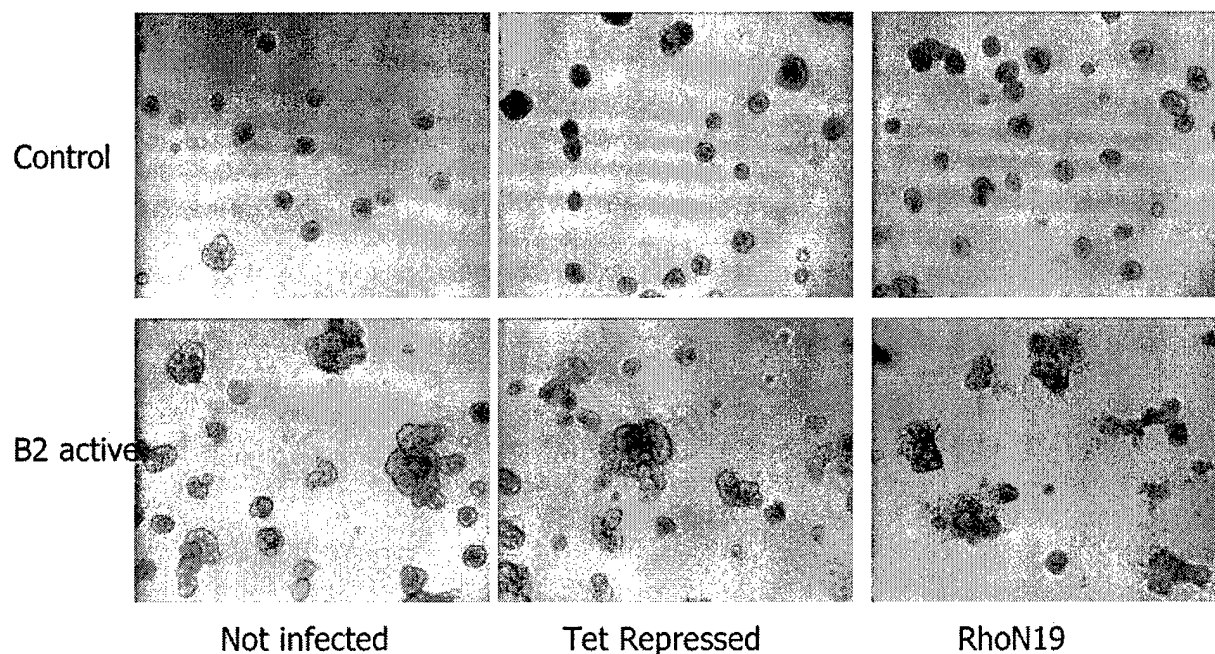
RhoN19 expression /ErbB2 activation

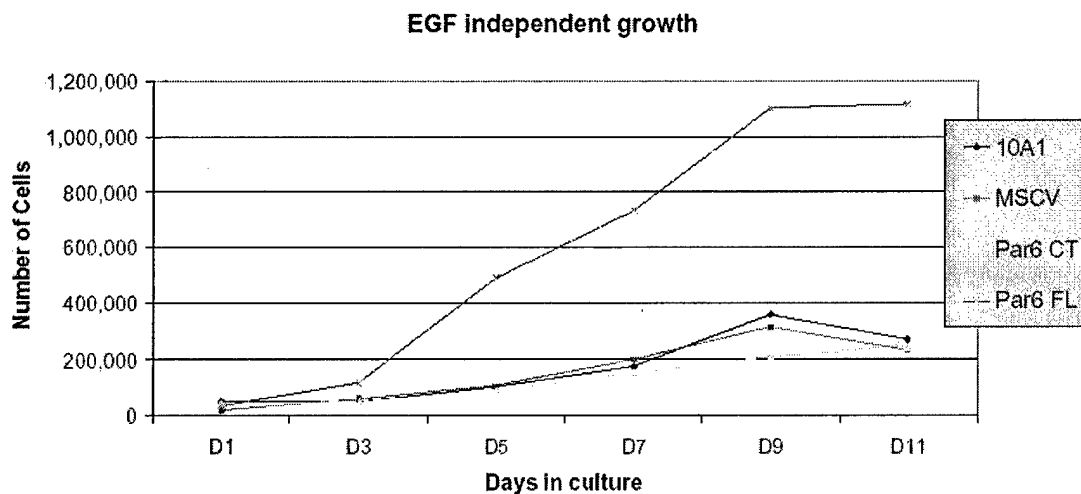**Figure 5.** RhoN19 expression disrupts architecture of acini structures when ErbB2 is activated.

Figure 6. EGF independent growth of MCF-10A cells expressing Par6 compared to parental, MSCV control and Par6 C-Term.

11